OPTIMIZATION OF CARDIOMYOCYTE DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS

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Bachelor's Thesis
November 2010
Degree Programme in Laboratory Sciences
Tampere University of Applied Sciences

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ABSTRACT

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Bachelor's Thesis, 50 pages, appendices 5 pages
November 2010

Human pluripotent stem cells can be differentiated into cardiomyocytes *in vitro*. Differentiated cardiomyocytes are at an important role in the future clinical applications of treating cardiac diseases. The purpose of this study was to optimize the cardiomyocyte differentiation of human pluripotent stem cells using four different culture media and to characterize the differentiated cardiomyocytes. The aim of the study was to find a best suited culture medium for cardiomyocyte differentiation.

Human embryonic stem cell line H7 and two induced pluripotent stem cell lines, h1/12 and 0/gG/25 were differentiated, and on day 22 the beating areas were counted. Cardiomyocytes were characterized by evaluating the beating rate and morphology, with immunocytochemical stainings and quantitative polymerase chain reaction (q-PCR).

The results show only small differences in the amount of beating areas acquired with each medium. 0% KO-SR hes medium gave the best result whereas the smallest amount of beating areas was acquired with Reges medium. The analysis with q-PCR showed positive gene expression of stem cell markers and high expression of cardiac markers in the differentiated cardiomyocytes with each tested medium and cell line. The highest gene expression in cardiomyocytes was observed in day 30 samples grown in 0%-medium.

According to the results, the best medium for the cardimyocyte differentiation would be 0% KO-SR hes medium. However, this medium should be studied even further with more different cell lines. Considering the q-PCR results, it would be interesting to study cardiomyocytes even older than 30 days, which are differentiated in 0% KO-SR medium.

Keywords: cardiomyocyte differentiation, stem cell, induced pluripotent stem cell, culture media, characterization, polymerase chain reaction

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1 INTRODUCTION

Stem cell research is one of the fastest growing and very promising field of research in natural sciences. Due to stem cell's potential to differentiate into any cell types, they make an excellent subject for medical research especially in regenerative medicine. With the introduction of induced pluripotent stem cells (iPSC) from human somatic fibroblasts, the possibilities for clinical usage of stem cells grew rapidly. Already nowadays stem cells are being differentiated into for example neural, mesenchymal and cardiac cells for research purposes. Nowadays with the massive increase in heart diseases, a great point of interest lies in the clinical exploitation of cardiomyocytes differentiated from iPS cells.

Cardiomyocytes cannot regenerate from cell damage after myocardial infarction. Being able to replace those damaged cells with cardiomyocytes differentiated from stem cells acquired from the patient would be a huge step forward in medical technology. So far cardiomyocytes have been differentiated as multicellular aggregates *in vitro*, but in the future it may be possible to grow new heart tissue. This bachelors thesis focuses on optimizing the growth media used in the differentiation of cardiomyocytes *in vitro*.

This bachelors thesis was done at the heart research group in Regea Institute for Regenerative Medicine. Regea is a joint institute under the administration of University of Tampere. The research there primarily focuses on stem cells and tissue engineering.

The purpose of this study was to test different cell culture media in the cardiac differentiation of pluripotent stem cells. In this study four different cell culture media were used and three different cell lines, two of which were iPS cell lines. Three of these cell culture media had not been used previously in Regea. One point of interest was to see whether the cell culture media used would affect embryonic stem cells and iPS cells differently. The aim of this study was to find a cell culture medium best suited for differentiation of cardiomyocytes, so that it could be used in the heart group of Regea for future research.

2 BACKGROUND

2.1 Stem cells

Stem cells are undifferentiated cells that have the ability to self-renew indefinitely in culture and are capable of differentiation. Human stem cells can be classified into four different groups: embryonic- (hESC), fetal-, adult- and induced pluripotent stem cells (iPS cells). HESCs are isolated from inner cell mass of surplus embryos obtained from fertilization treatments First hESC line was derived in 1998. (Thomson & Co 1998.) Adult stem cells are responsible for tissue regeneration and fetal stem cells are involved in the growth of organs in the developing fetus. Adult and fetal stem cells can differentiate only into some specific cell types, thus they are called multipotent. HESCs and iPS cells are pluripotent stem cells, meaning that they can differentiate into almost any cell type, excluding extra embryonic tissue and the placenta. Because of their ability to differentiate into various cell types, stem cells are currently one the most studied subjects in molecular biology. (Passier & Mummery 2003.)

The derivation of hESC line is carried out by first removing the zona pellucida and trophoblast from 5-7 days old blastocyst. The remaining inner cell mass is transferred to mitotically inactivated feeder cells, normally human foreskin fibroblasts (figure 1). After transfer the cells are cultured in culture medium containing growth factors and nutrients for the cells. However, because the feeder cells are derived from primary tissue, their lifespan is relatively short and that is why the feeder cell layer has to be replaced regularly. (Unger & Co 2009.)

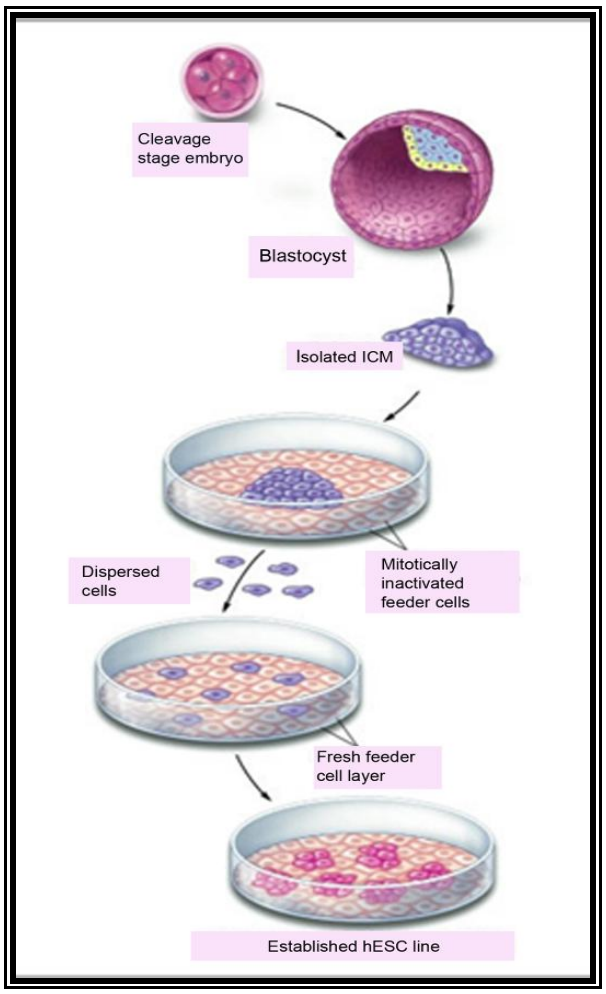


FIGURE 1. The derivation of human embryonic stem cells (figure modified from Vaajasaari 2010).

Pluripotent stem cells express certain genes which maintain them in an undifferentiated state. Such markers are OCT3/4 (also called POU5F1), Nanog, Sox2 and FoxD3, for example. Oct3/4, a transcription factor gene is especially highly expressed in stem cells. Identifying these markers from cells under research confirms them as stem cells. (Sperger & Co 2003.)

2.2 Induced pluripotent stem cells (iPSC)

iPS cells are the newest discovery in the field of regenerative medicine. iPS cells are derived from somatic cells that are genetically reprogrammed, or induced, into a pluripotent state. By inducing the somatic cells with specific genes, they are forced to express genes and factors that make them return to stem cell-like state. iPS cells have been found to express stem cell markers and to differentiate into cells of all three germ layers: ectoderm, mesoderm and endoderm. First iPS cells were derived from mouse fibroblasts in 2006 and a year later, from adult human fibroblasts. (Takahashi & Co 2007.)

Generating iPS cell lines requires four transcription factor genes: OCT3/4, Sox2, Klf4 and c-Myc. This combination was described by Doctor Yamanaka in 2007. These exogenous genes are delivered virally to fibroblasts and the cells are grown on hESC culture conditions. This will cause the fibroblasts to transform into iPS cells, which are very similar to hESCs in morphology, proliferation and gene expression (figure 2). The differentiation capacity seems to be similar to hESCs. (Freund & Mummery 2009.)

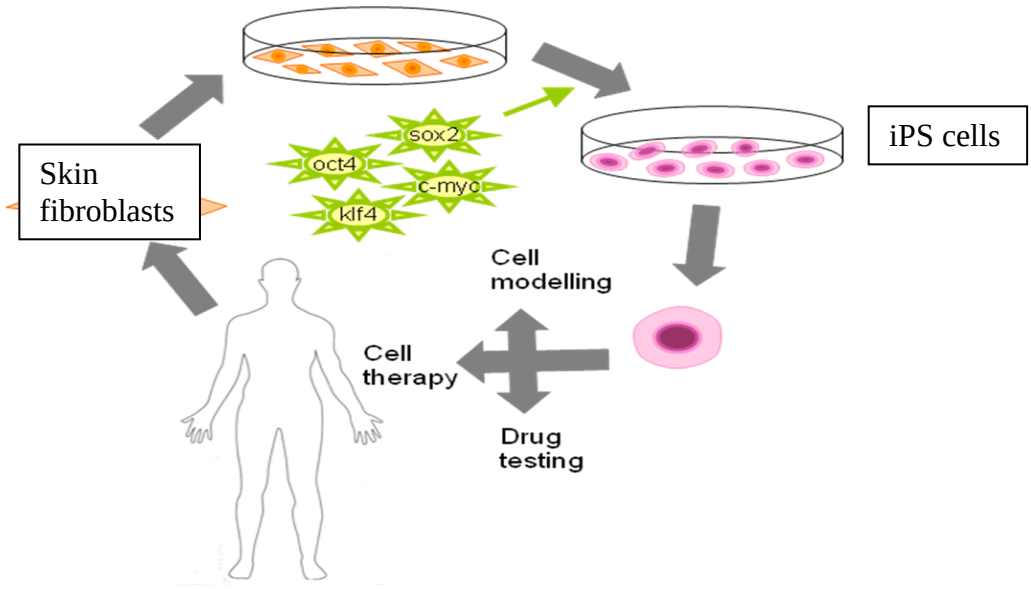


FIGURE 2. The induction of iPS cells from human adult fibroblasts (figure modified from Lahti 2010)

With iPS cell technology it is possible to create patient-specific stem cell lines with a rare disease for example. This will provide cells for future therapy without the need for immune suppression and it will also give the scientists a new way to study stem cells carrying disease traits. (Freund & Mummery 2009.)

2.3 Stem cell culture

Culturing of human stem cells is a very delicate process and prone to contamination, like any other cell culture. The goal of stem cell culturing is to keep the cells alive and in an undifferentiated state. The common way of culturing stem cells is to use feeder cells. Feeder cells secrete growth factors that induce the proliferation of stem cells and prevent differentiation. To be useable, feeder cells have to be inactivated with irradiation or with a treatment with mitomycin C to stop them from dividing. In this study, mitotically inactivated mouse embryonic fibroblast cells (MEFs) were used as feeder cells. (Allele Biotechnology 2010.) Mitomycin C is a strong antibiotic with antitumor activity against various types of mouse, rat, hamster and also human cancers. Mitomycin C also prevents the synthesis of DNA in bacteria and cultured human cells but not the synthesis of RNA or protein translation. This ensures cell growth but prevents cell division. (Kuroda & Furuyama 1963.)

Cells are cultured in gelatin coated well plates to ensure their attachment to the bottom of the well. Feeder cells are plated at least one day before transferring stem cells on top of feeders. Stem cells grow and fill the well typically within a week, thus making it a necessity to divide stem cells once a week. The medium normally used for undifferentiated stem cells is called KSR medium. KSR is a serum free medium which contains 20% knockout serum replacement and bFGF growth factor. (Inzunza & Co 2004.)

Fetal bovine serum (FBS) is extracted from the blood of bovine fetuses and has been used for a long time in cell culture. The fact that the serum comes from animals makes it

unsuitable in future clinical applications of cardiac cells, but it is still a widely used ingredient in stem cell research. (Jochems & Co 2002.) FBS is used in cell culture because it contains many growth factors and very little antibodies. These growth factors in FBS enhance the differentiation of stem cells which is the reason that FBS has been replaced in the culture of undifferentiated stem cells. There are also studies that suggest better cardiac differentiation in serum free cultures compared to cells grown in culture containing 20% or less FBS. (Passier & Co 2005.)

Knockout serum replacement (KO-SR) is a serum free formulation, designed to replace the FBS as a supportative factor in the growth of undifferentiated stem cells. Stem cells grown in KO-SR enhanced media are discovered to differentiate much less and to grow better than cells grown in FBS enhanced media. Using a serum-free supplement also minimizes the risk of pathogen and foreign animal protein contaminations in medium. (Inzuza & Co 2004.)

2.4 Cardiomyocytes

Cardiomyocytes are the main cell type of myocardium tissue. Although not the most numerous, they cover over 90% of the myocardium volume. Cardiomyocytes are continuously contracting muscle cells and almost completely dependent on aerobic metabolism, which is why they contain almost 25% mitochondria of the total cell volume. The contractile unit in cardiomyocyte is the sarcomere, which consists of myosin and actin filaments and regulatory proteins like troponin and tropomysin. Cardiomyocytes comprise about 25% of the total cells in heart, the rest are endothelial cells like fibroblasts and cells constructing the myocardial capillary system. There are different kinds of cardiomyocytes: atrial, ventricular and nodal. Atrial myocytes are located in the atrium where blood is collected and ventricular myocytes in the ventricle, from where the blood is pushed out of heart. Cardiomyocytes contain also much of sarcoplastic reticulum, a special form of smooth endoplastic reticulum, which function is to store and release Ca^{2+}-ions needed during muscle contraction. (Humpath.com 2010.)

2.4.1 Differentiation of cardiomyocytes

During early embryogenesis the endoderm is known to affect greatly in the cardiac differentiation of the precursors in the adjacent mesoderm. When this fact was discovered, two different approaches for the differentiation of cardiomyocytes from hESCs were developed. The first one was a co-culture with mouse visceral endoderm-like cells (END-2). END-2 cells produce cardio-inductive signals to hESCs cells. The signaling is thought to originate from cell-to-cell interactions and/or certain stimulating factors secreted by END-2 cells, but the effects of END-2 cells in cardiac differentiation are indisputable. (Vidarsson, Hyllner & Sartipy 2010.)

The second method for differentiation was addition of hormones and growth factors directly to hESCs grown as multicellular aggregates called embryoid bodies or in a monolayer fashion. Stem cells require also certain growth factors to remain in an undifferentiated state. Such growth factors are basic fibroblast growth bFGF and activin a, for example. The interesting part about these growth factors is that some of them also enhance differentiation of stem cells, with the right concentration and timing in the culture medium. With the removal and alteration of concentrations of such factors from culture medium and implication of differentiation enhancing growth factors, researchers can differentiate stem cells practically into any desired cell type. (Singla & Burton 2005.)

2.4.2 Growth factors

Growth factors enhancing cardiac differentiation can be classified into three major families of growth factors: the transforming growth factor (TGF) β-family, the Wnt family and fibroblast growth factor family (FGF). (Vidarsson, Hyllner & Sartipy 2010). TGF-β-family includes bone morphogenic proteins (BMPs) and activin. BMPs have an important role in early embryogenesis and induction of cell differentiation. Activin-A is a protein that stimulates the release of follicle stimulating hormone and it has wide

variety of biological activities, most important ones being mesoderm induction and cell differentiation. (R&D Systems 2001.)

Wnts are secreted glycoproteins that are important in the development of *Drosophila*, but Wnt11 protein has also been found to increase cardiac gene marker expression in cell cultures treated with Wnt11 conditioned medium. (Singla & Burton 2005.)

Fibroblast growth factors are polypeptides from a family of cytokines comprising of 23 members. All of those molecules interact with FGF receptors and that complex is involved in many biological activities, including embryonic development, cell migration and tumor growth. FGF2 has been found to increase cardiac differentiation in hESCs. (Singla & Burton 2005.)

2.5 Characterization of cardiomyocytes

2.5.1 Gene expression of cardiomyocytes

Cardiac cells express certain genes unique to them, which allows the identification of cardiomyocytes with some molecular biology techniques. Such genes are Troponin T, a part of tropomyosin complex which works with actinin in heart muscle cells, MYH7, which codes for the production of myosin heavy chain protein (Megy & Co. 2002.), Nkx2,5 a gene required for cardiac development in vertebrate animals, which is most active in early cardiac progenitor cells (Schwartz & Olson 1999.) or ISL1, a transcription factor required for the development of complete heart in the cardiac mesoderm. (Cai & Co 2003.)

2.5.2 Immunocytochemical staining

Immunocytochemical staining is a basic biochemical method to visualize a specific protein or even tissue in a sample using fluorescently labeled antibodies. Antibodies, or immunoglobulins, are glycoproteins found in blood that recognize and neutralize foreign objects. Antibodies bind to their specific target molecule, called the antigen. Antigens are the locations in samples that are points of interest in immunocytochemical staining procedure. When antibodies bind to their target site the desired target can be observed with a fluorescence microscope. (Campbell & Farrel 2008, 417-423.)

There are two possible ways to use antibodies in immunocytochemical staining: single staining with only one antibody or double staining with two different antibodies. In double staining the primary antibody binds to the target site in the sample after which the fluorescently labeled secondary antibody is added. Secondary antibodies are targeted against the primary antibodies, which allow them to bind to the primaries. The staining color comes from fluorescent secondary antibodies. The antibodies are produced in different animals like mouse, rabbit, donkey and goat. The secondary antibodies are always produced in different animals than primary ones. For example if the primary antibody is rabbit IgG, a common antibody, the secondary antibody should be anti-rabbit IgG and cannot be produced in a rabbit. (IHCWORLD 2007.)

2.5.3 Polymerase chain reaction

Polymerase chain reaction, PCR for short, is a technique used to efficiently amplify a desired DNA sequence. The DNA sequence to be amplified can be any desired sequence of the sample DNA. PCR reaction amplifies DNA exponentially with the help of nucleotides, primers and thermostable polymerase enzymes. The primers are short segments of DNA with the known amino acid order and polymerase enzymes were found from archae in hot springs and other extreme locations and are still in use today. Commonly used polymerase is Taq-polymerase, derived from *Thermus aquaticus*, a bacterium living in hot springs. (Campbell & Farrel 2008, 386-388.)

For the PCR reaction a master mix solution is prepared. Master mix contains all the necessary ingredients for the reaction. Those are buffer solution, nucleotide mixture, called the dNTP mix, polymerase enzyme, water as solvent, RNase inhibitor and the assay. The assay is the primer for the gene of interest. The master mix is added in excess to all samples so that the wanted gene can multiply exponentially. (Campbell & Farrel 2008, 386-388.)

PCR consists of three stages: denaturation, annealing and extension. In denaturation stage the temperature is raised to +94°C for few minutes. This causes the DNA strands to denature and separate from each other. In the annealing step the temperature is lowered to about 55°C where the specifically designed primers bind to their complementary places in the template DNA. Last step is extension where nucleotides start to add to the growing 3' end of DNA strand starting from the primer. One PCR cycle consists of these three stages and once there has been enough cycles, there is enough of the desired DNA material. Normally 25-35 cycles are enough to produce a satisfying amount of DNA. Figure 3 visualizes the exponential amplification of the target gene in the sample. At the end of PCR cycles the DNA yield can be measured with gel electrophoresis, for example. (Campbell & Farrel 2008, 386-388.)

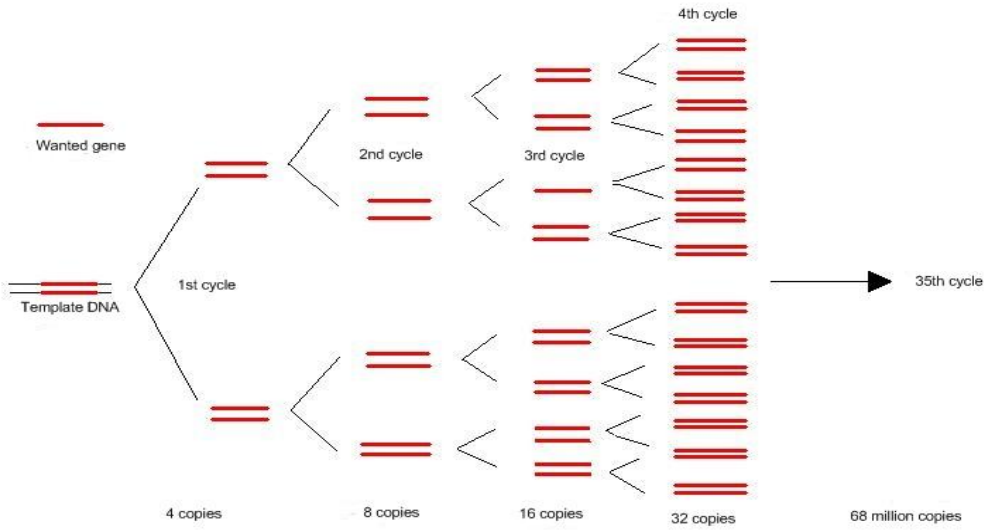


FIGURE 3. Polymerase chain reaction, amplification of the target gene (Haponen 2010)

2.5.4 Quantitative polymerase chain reaction

Quantitative PCR (q-PCR) is also called real time- PCR because the rate of reaction can be followed in real time. Q-PCR uses fluorescent probes to detect the amount of target sample. The probes can be non-specific or specific, in which case more than one sample can be studied simultaneously. Fluorescence markers bind to multiplied DNA segments and emit fluorescence once bound. (Wilson & Walker 2005.)

The most commonly used non-specific marker is SYBR Green I. It is a dye that binds to double stranded DNA and emits fluorescence at 520nm. The intensity of fluorescence is proportional to the amount of DNA in which the dye can bind to. Therefore an increase in fluorescence is observed after each cycle. SYBR markers use normally two primers: forward and reverse primers. Forward primer starts to build DNA strand from the beginning of the target sequence and reverse primer starts from the end of the sequence and codes the addition of nucleotides backwards. (Wilson & Walker 2005.)

For specific quantification there are double-dye probes called TaqMan probes. These probes are small nucleotide sequences complementary to one of the strands of desired DNA, the amplicon. TaqMan probes contain a fluorophore and a quencher at 5’ end which prevents emission from the fluorophore. Once the probe binds to DNA and polymerase starts to build a new DNA strand, the 5’end of probe is degraded and the quencher is released in the solution thus allowing the fluorophore to emit fluorescence, which is also proportional to the amount to DNA amplified. (Eurogentec 2010.)

To identify the DNA products after the amplification, a dissociation curve analysis technique can be used. This technique adds a dissociation stage after the PCR amplification cycles. Dissociation stage consists of three stages as seen in table 1.

TABLE 1. Dissociation curve analysis stages

Temperature	95°C	60°C	95°C
Time	15 sec	1 min	15 sec

The dissociation temperature of the amplified product gives a DNA melting curve, which expresses the GC/AT ratio of DNA and length of DNA strand. This gives information about the sequence of the DNA strand and with this information undesired PCR products can be distinguished from target products. This removes the need to run gel electrophoresis after PCR run. (Ririe & Co 1997.)

After the q-PCR run the results are analyzed using the relative quantification of gene expression data and the $2^{-\Delta\Delta C_T}$-method as described by Livak and Schmittgen (2001, 402-408.) This method relates to the PCR signal of the target sample to a reference sample called the housekeeping gene. This allows the visualization of relative changes in gene expression between different samples. The housekeeping genes are called RPLP0 for TaqMan assays and PPIG for SYBR assays.

2.5.5 Reverse transcription PCR

Sometimes the sample is composed of RNA instead of DNA. In that case a complementary DNA copy has to be made out of sample RNA. This is done using reverse transcriptase enzyme. Reverse transcriptase is obtained from viruses and it functions as a RNA dependant DNA polymerase. Once the RNA has been transcribed, the PCR or q-PCR reaction can be carried out normally. (Campbell & Farrel 2008, 384-386.)

3 THE PURPOSE AND AIM OF THE STUDY

The purpose of this bachelor's thesis was to test four different cell culture media in the cardiac differentiation of pluripotent stem cells and to characterize the differentiated cardiomyocytes. The characterization was carried out by calculating the beating cardiomyocyte areas from the culture wells and by evaluating their morphology and beating rate. The identification of heart muscle cells and especially ventricular heart muscle cells from other cells was done by immunocytochemical staining. The expression of genetic cardiac markers from the cardiomyocytes was characterized with quantitative polymerase chain reaction.

The aim of the study was to find a best suited cell culture medium for cardiomyocyte differentiation. So far the heart group in Regea has used 10%-KO-SR medium for cardiac differentiation. Along with the old medium, three other media were used, two of which were serum free and one xeno-free. The optimal differentiation medium would produce a good percentage of beating areas with a healthy rate of beating and the cells expressing all the wanted cardiac gene markers. The aim of the study was also to find cheaper medium for cardiac differentiation in Regea, to ease the reagent costs in research.

4 MATERIALS AND METHODS

4.1 Cell lines used in study

In this study three different cell lines were used: a commercial human embryonic stem cell line H7, one patient iPS cell line called 0/gG/25 and a iPS cell line derived from neonatal fibroblasts called h1/12. 0/gG/25 cell line is derived from the skin fibroblasts of a patient with a known disease. The effects of those diseases are not included in this project though. H7 is a commercial cell line used as a control in Regea and in many other institutes.

4.2 Culture media used in study

This study was carried out using four different cardiac differentiation media: 0% KO-SR hes, 10% KO-SR hes, EB-medium and Reges (Appendix 1). All the media contain the same basic ingredients with minor alterations in quantities. 0%- medium is relatively plain medium with just base Knockout DMEM medium and some obligatory supplements which are listed in table 2. Normally ascorbic acid is added to 0%-medium, but it was left out in this study. This medium is completely serum- and serum replacement free. 10% KO-SR contains 10% knockout serum replacement supplement. This is the medium currently in use in Regea's heart group. EB-medium contains 20% FBS which makes it unsuitable in future clinical applications of cardiac cells, but it is still an important ingredient in stem cell research.

The most interesting medium to be tested is the Reges medium, which is entirely developed in Regea institute. Reges is a serum- and xeno-free medium which means it contains nothing extracted from animals and thus could be used with cells for clinical

applications. Although the same is true for 0% hes-media, it is interesting to see how this newly developed medium works. The components of Reges are very similar to 10% KO-SR, with some differences shown in the table 2. KO-SR contains all the components in the reagent table listed in appendix 1, except bFGF, Activin-A and Retinol and the other components used in every media.

TABLE 2. Reagent differences in the four test media

Reagent	0 %	10 %	EB	Reges
KO-DMEM	x	x	x	x
KO-SR		x		
FBS			x	
NEAA	x	x	x	x
Glutamax	x	x	x	x
Pen/Strep	x	x	x	x
b-mercaptoethanol	x	x		x
bFGF				x
Activin a				x
Retinol				x
Other Reges components				x

4.3 Differentiation procedure

To differentiate stem cells into cardiomyocytes the cells were transferred into 12-well plates which already contained END-2 cells. From the 6-well plates where the stem cells were growing on, the MEF layer was removed and the remaining cells were scraped from the bottom. Then about 30 cell flakes per well were pipetted onto END-2 cells in 12-plates. Prior to cell transfer the END-2 plates had been filled with 0% KO-SR hes-medium with ascorbic acid.

Cells were incubated in 0% KO-SR hes-medium for 15 days with medium changes at day 6, 9, and 12. At day 15 the new test media was replaced onto the cell plates. After day 15 the medium was changed 3 times a week. The differentiation procedure was done from two different passages of each cell line.

4.3.1 Identification and evaluation of beating areas

Cells were monitored at day 22 from the start of differentiation and beating areas were counted and their morphology evaluated using a regular light microscope and a heating plate. A beating area on the plate is an aggregate of cardiac cells differentiated from stem cells. The size, shape and location of beating area may vary even between cells of the same line and with the same growth medium. The cells were observed using a 4 times magnification of the microscope.

4.3.2 Dissociation of beating areas

On day 23 beating areas of cardiomyocytes were cut manually with a sterile scalpel. The beating areas were transferred into one well in 24-well plate in their own culture medium. After that the beating areas were dissociated in three dissociation buffers (appendix 2). The buffers are called 1, 2 and 3, and the incubation time was 30min, 45min and 1h, respectively. Incubations 1 and 3 were carried out in room temperature and incubation 2 in +37°C incubator. After the third incubation the cells were transferred to EB medium and pipetted up and down to break the cell clumps into single cells. The cell solution from each medium was divided into four wells and EB medium was added to the total volume of 1ml. The dissociated cells were left to attach and grow in an incubator for 5-7 days after which they were used for immunocytochemical staining.

4.4 Immunocytochemical staining

In this study a double staining technique was used and the three pairs of primary antibodies were: Connexin-43 + α-Actinin, ventricular MHC + Troponin T and Troponin T + Vimentin. Connexin-43 binds to muscle cells and α-Actinin binds to thin

filaments in striated muscle cell called the Z discs. (Young & Co 1998.) Connexin-43 also connects to Ca^{2+}-channels in muscle cells (Heino & Vuento 2004, 292). Ventricular MHC stands for myocin heavy chain, a protein in heart and skeletal muscle responsible for motor actions. MHC antibody binds to ventricular muscle cells. (Kolossov & Co 2005.) Troponin T binds to heart muscle cells and Vimentin binds to every other cell types. (R&D Systems 2010.)

Immunocytochemical staining was done from dissociated cells according to the figure 4, which illustrates the process. Table 3 lists the reagents used in the protocol to clarify the picture. On the plate were 4 wells for each test medium and three pairs of antibodies, so one set of wells was a spare. Antibodies were added to wells in the following order: Connexin + α–Actinin, MHC + Troponin T and Vimentin + Troponin T, and the well numbers were 1, 2 & 3, respectively. The corresponding secondary antibodies were added after primary antibodies were incubated overnight. List of primary and secondary antibodies and their dilutions are listed in the appendix 3. Vectashield mounting medium was added at the end of procedure to help retaining fluorescence and to give anti-fading ability to the fluorescent colours. Vectashield also contains DAPI counterstain, which stains the nucleus of the cells. (Vector Laboratories 2010)

TABLE 3. Reagents used in the double fluorescence protocol

Abbreviation	Reagent
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
BSA	Bovine Serum Albumine
NDS	Normal Donkey Serum
PB	Phosphate buffer

In table 4 are listed the contents of different solutions used.

TABLE 4 Solutions for immunocytochemical staining

Blocking solution	10% NDS, 01% Triton-X, 1% BSA
Primary mix	1% NDS, 0,1% Triton-X, 1% BSA + antibodies
Secondary mic	1% BSA in PBS + antibodies

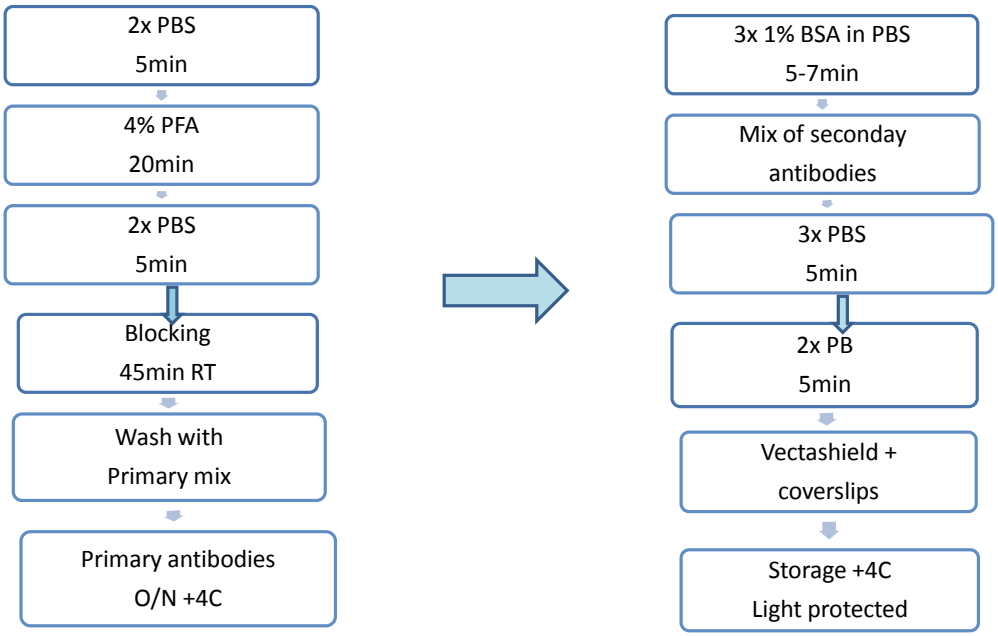


FIGURE 4. Scheme of immunocytochemical staining procedure

The secondary antibodies were fluorescent on different wavelengths, so when observed with a fluorescence microscopy, differently coloured antibodies could be visualized by changing the wavelength of the light source. Cells were observed with 20x magnification, and the pictures taken were piled together with computer software to acquire an image with three different colours.

4.5 RNA extraction

RNA samples were taken from the differentiated cardiomyocytes on days 0, 13, 22 and 30. For the samples two random wells from a 12-well plate were scraped with a cell scraper and washed once with phosphate buffered saline, PBS. The cells were transferred to an eppendorf tube and the cell debris was spinned down. PBS was sucked

out and 350µl of RA1 buffer was added to lyse the samples. The samples were stored in -80°C until RNA extraction was carried out.

For the extraction of RNA from the sample, a commercial Cytospin kit was used. According to manufacturer's protocol, the samples were filtered with a centrifuge and filter membrane, bound to RNA columns and desalted with membrane desalting buffer. The DNA was removed enzymatically with DNase, an enzyme which hydrolyses the phosphodiester bonds in DNA strands. The remaining RNA was washed three times with manufacturers wash buffers and the RNA was eluted in RNase free water, 60µl per sample. (Macherey & Nagel 2010). The extraction protocol is illustrated in figure 5.

After the extraction the purity of the samples was measured with Nanodrop spectrophotometer and the samples were stored in -80°C freezer. The purity was analyzed with RNA concentration ratio from two different wavelengths measured with the spectrometer: 280nm/260nm. When the concentration ratio is near 2,10 the RNA is considered pure. (Biomedical Genomics 2007)

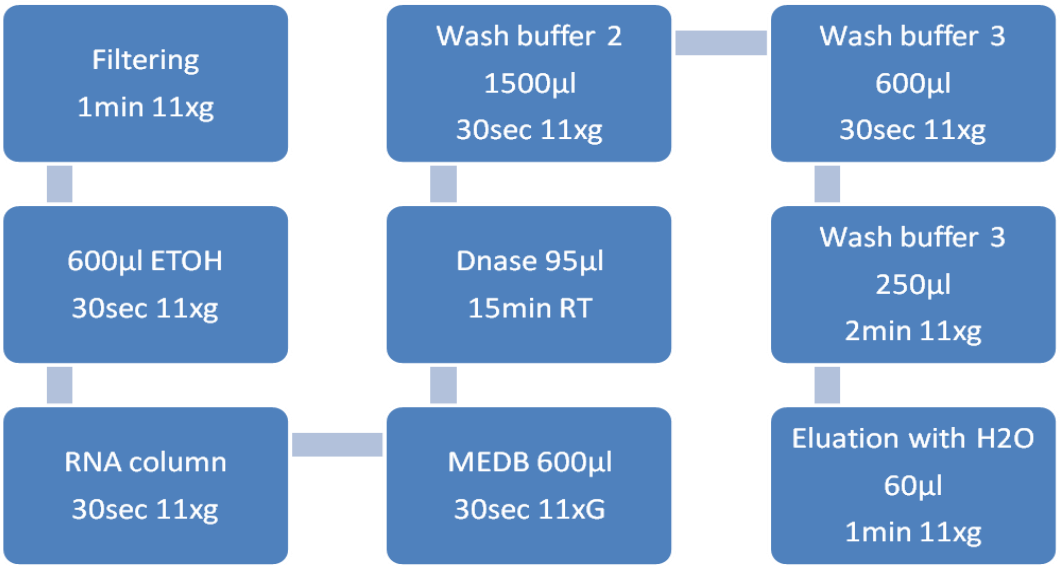


FIGURE 5. RNA extraction protocol

4.6 Complementary DNA reverse transcription

Extracted RNA from samples was transcribed to complementary DNA using reverse transcriptase enzyme. The reaction was carried out in normal PCR machine. The samples were kept on ice during the working procedure. First the samples were diluted with RNase free water to achieve a RNA concentration of 1mg/10µl. Every sample had a duplicate sample and RNA was pipetted from every two parallel samples into one tube to get the desired concentration of 1mg/10µl. Appendix 4 shows the amounts of RNA in samples and the amount pipetted to cDNA samples.

Master mix solution was prepared according to work protocol (table 5) and 10µl was added to each sample tube. The solution in tubes was spinned down with centrifuged and moved to the PCR machine. PCR conditions are listed in table 6.

TABLE 5. Master mix reagents

Reagent	Amount µl (one reaction)
10x Rt Buffer	2
25x dNTP mix	0,8
10x random primers	2
Reverse transcriptase	1
RNase inhibitor	1
H_2O	3,2

TABLE 6. PCR run conditions

Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 sec	∞

After the PCR the samples were stored in -4°C freezer until the q-PCR run.

4.7 Quantitative polymerase chain reaction

Before the reaction, half of the cDNA sample volume was diluted 1:3 and this dilution was used for the q-PCR reaction. For the characterization of the cell samples various

gene markers were used. The markers are listed in table 7 as well as the visualization technique that was used. Each gene marker had to be made in separate plates.

TABLE 7. Gene markers used in the study.

Gene	Visualization technique
Nanog	TaqMan
OCT3/4 (POU5F1)	TaqMan
RPLP0 (housekeeping gene)	TaqMan
PPIG (housekeeping gene)	SYBR
Troponin T	SYBR
Nkx2,5	SYBR
ISL-1	SYBR

The master mix solution was a ready-to-use commercial solution. For one sample 7,5µl of Master mix was used. 5,75 µl of water and the primers were added to master mix solution to get the working solution. 0,75 µl of TaqMan primers or 0,45 of forward and reverse SYBR primers were added. Master mix was pipetted onto 96-well plate, 14 µl per well according to premade pipetting charts (figure 6). Water and –RT samples were added as controls. –RT is a normal sample from cDNA reverse transcription reaction, but it didn't contain the enzyme and therefore shouldn't contain any DNA. Each sample was made in triplicate for the q-PCR to make sure of successful reaction in case of contaminations or accidental pipetting faults. 1 µl of sample was added to each well with the master mix and the top of plate was sealed with a plastic sheet cover. The plate was centrifuged and placed in the q-PCR machine. The thermal program was premade in the machine. Table 8 shows the thermal program of the TaqMan assay and table 9 shows the SYBR assay.

TABLE 8. Thermal program of q-PCR reaction for TaqMan assay

Temperature	50 °C	95°C	95°C	60°C
Time	2 min	10 min	15 sec	1 min
Cycles	1	1	40	

TABLE 9. Thermal program of q-PCR reaction for SYBR assay

Temperature	50 °C	95°C	95°C	60°C	95°C	60°C	95°C
Time	2 min	10 min	15 sec	1 min	15 sec	1 min	15 sec
Cycles	1	1	40		1	1	1

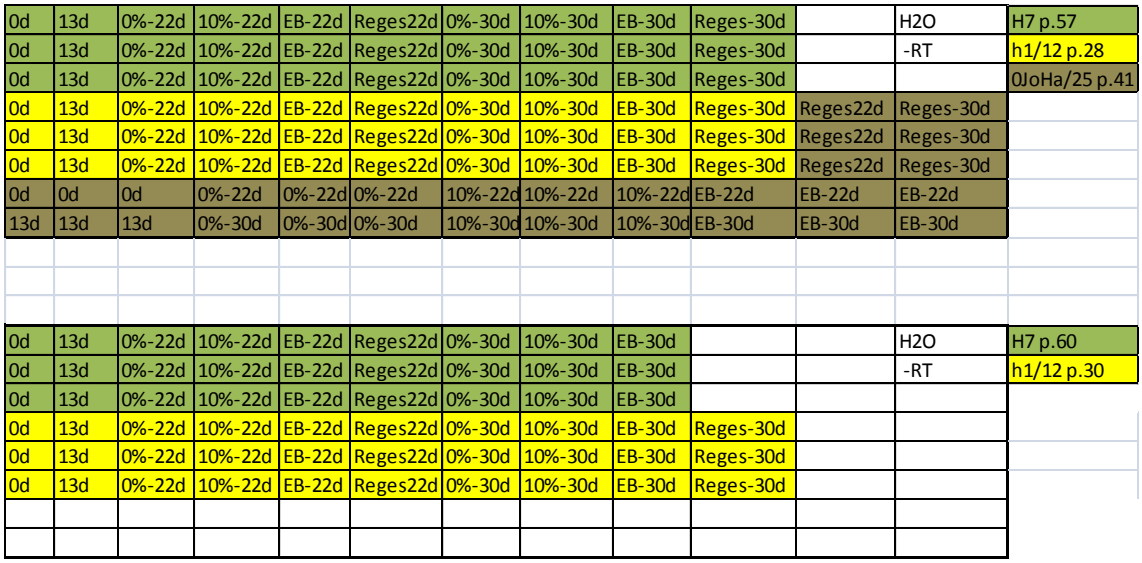


FIGURE 6. The pipetting chart for samples in q-PCR

After the q-PCR runs the results were analyzed using the relative quantification of gene expression data and the $2^{-\Delta\Delta C}{}_T$-method. The purpose of q-PCR was to show the changes in gene expression from day 0 through days 13 and 22 to day 30. As previously stated, Nanog and OCT3/4 are stem cell markers, which means that their expression should be high on day 0 samples, when the sample cells were still undifferentiated stem cells. The other markers, ISL1, NKX2,5 and Troponin T are cardiac markers and should be expressed during and after the differentiation.

5 RESULTS

5.1 Beating areas

Every media tested in the study yielded beating areas and therefore worked in the differentiation of stem cells into cardimyocytes, but the amounts of beating areas and they quality varied with each medium. The best percentage of beating areas per attached cell colony was achieved with 0% KO-SR hes-medium. The worse medium percentage-wise was Reges medium, although the differences are very small. The amount of beating areas, attached cell colonies and beating percentage for each cell line and passage number is illustrated in tables 10, 11 and 12. The overall differentiation results are illustrated in table 13 to clarify the results between each medium.

TABLE 10. Differentiation results from H7 cell line

H7 p.57			
Medium	Attached colonies	Beating areas	Differentiation-%
0 %	368	113	**31**
10 %	338	188	**56**
EB	383	138	**36**
Reges	390	113	**29**
H7 p.60			
Medium	Attached colonies	Beating areas	Differentiation-%
0 %	331	53	**16**
10 %	361	35	**10**
EB	405	35	**9**
Reges	387	20	**5**

TABLE 11. Differentiation results from h1/12 cell line

h1/12 p.28			
Medium	Attached colonies	Beating areas	Differentiation-%
0 %	271	38	**14**
10 %	264	45	**17**
EB	299	52	**17**
Reges	289	30	**10**
h1/12 p.30			
Medium	Attached colonies	Beating areas	Differentiation-%
0 %	344	75	**22**
10 %	488	43	**9**
EB	277	38	**14**
Reges	409	63	**15**

TABLE 12. Differentiation results from 0/gG/25 cell line

0/gG/25 p.41			
Medium	Attached colonies	Beating areas	Differentiation-%
0 %	398	48	**12**
10 %	377	18	**5**
EB	332	28	**8**
Reges	373	15	**4**
0/gG/25 p.44			
Medium	Attached colonies	Beating areas	Differentiation-%
0 %	57	2	**4**
10 %	92	2	**2**
EB	57		**0,0**
Reges	76	2	**3**

TABLE 13. Overall differentiation results from each medium

	0 %	**10 %**	**EB**	**Reges**
Attached colonies	1769	1920	1753	1924
Beating areas	329	331	291	243
Differentiation-%	**18,60**	**17,24**	**16,60**	**12,63**

The last test for 0/gG/25 p.44 yielded unsatisfactory results, which was caused by the strangely small amount of cells attached to the plate at the beginning of differentiation procedure. There were only a few attached cell colonies per well which of course made it much harder to get beating areas from that cell mass. The differentiation procedure was carried out the same way as all the previous ones and despite the results, no

contaminations were found from the wells. Because of the low amount of cells, 0/gG/25 p.44 cell line was not included in the rest of the characterization analysis.

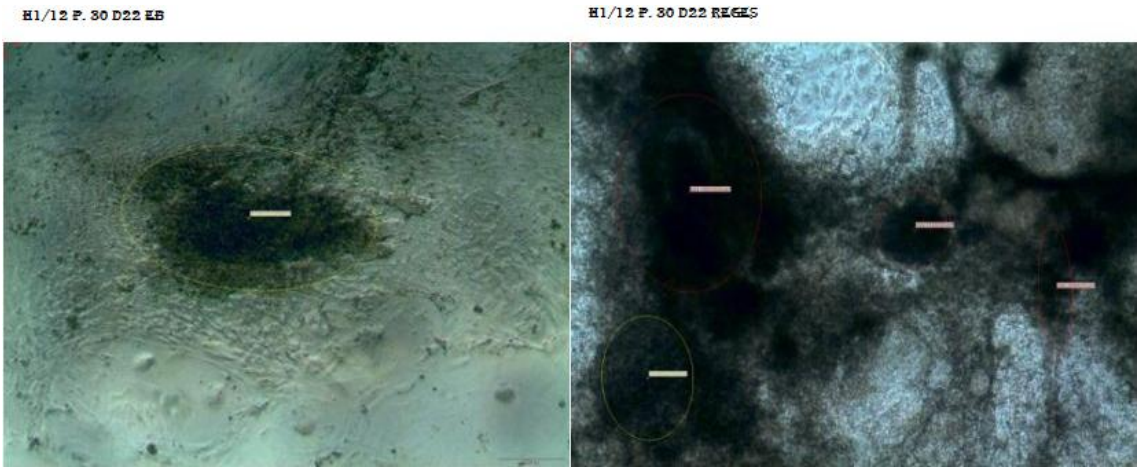
5.1.1 Cell morphology and beating character of differentiated cardiomyocytes.

The most interesting effect on the growth and differentiation of the cell lines was with 0% KO-SR medium. Soon after the medium change on day 15 the beating areas started to appear but at the same time the undifferentiated cell mass started to detach from the bottom of the wells. As a result on day 22 when beating areas were counted most of the cell mass was gone or floating in the medium as dead cell debris, but the beating areas were looking really good and beating nicely. With the three other media the cell mass on the wells looked normal and healthy, cells started to detach only with 0% KO-SR medium. Picture 1 shows this phenomenon with a beating area from 10% KO-SR medium as reference.

PICTURE 1. Beating areas from day 22 0/gG/25 p.41

The morphology of beating areas was pretty similar with 0% KO-SR- and10% KO-SR-media. Most beating areas were large ranging from 200 to 1000 µm and their shape was mostly roundish. EB- and Reges-media yielded as large beating areas as 0%-and 10% KO-SR but also many small beating areas, even areas the size of few cells. There was

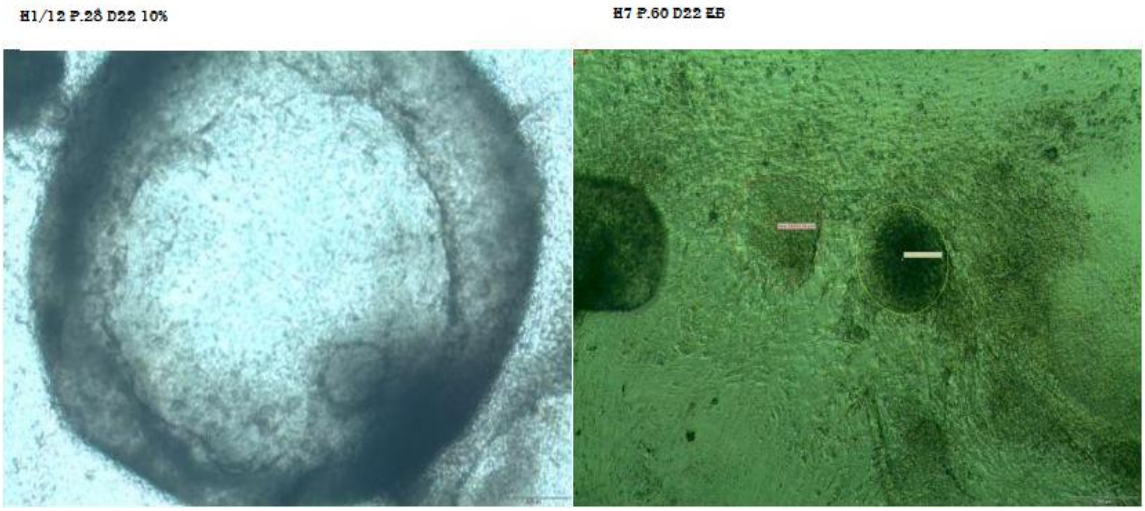
no distinguishable difference in the shapes of the beating areas between the four test media. Picture 2 illustrates good examples of beating areas with EB and Reges media from d22 h1/12 p. 30 cell line.



PICTURE 2. Beating areas from d22 h1/12 p.30

The rate of beating with 0%-, 10%- and EB-medium was mostly consistent with the average beating rate of 30-60 beats/min with few exceptions that are normal in this line of research. Out of these three, cells grown in EB medium showed the largest variety in the speed of beating. The interesting part was the beating rate acquired with Reges medium. This medium caused really unstable beating. Some areas were beating really fast and some slower but the most common were the long pauses between beating cycles. For example the cells beat about ten times followed by a pause of 30 seconds after which another cycle of ten beats followed. These pauses between beatings were manifested in all three cell lines and passages but not in every possible beating area.

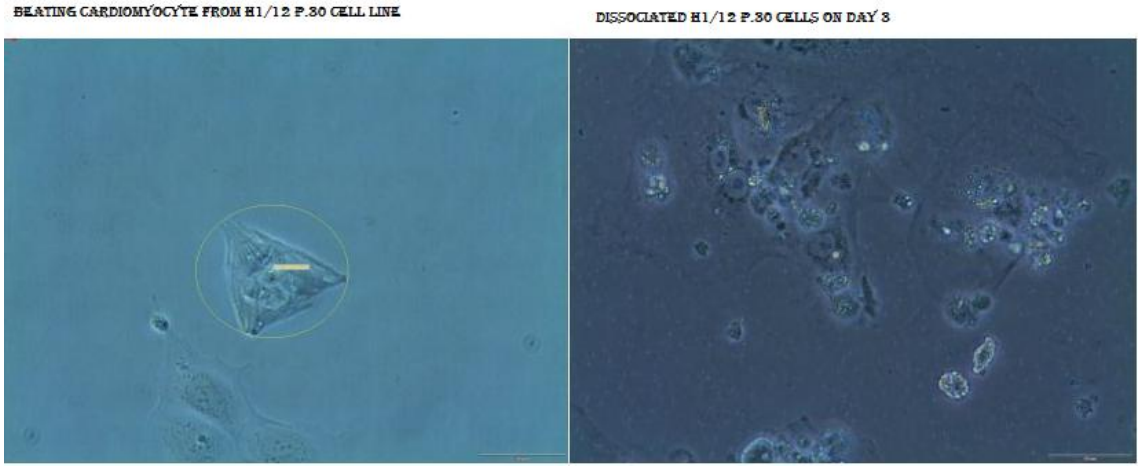
Beating areas were formed mostly in aggregate formations in the wells, but some beating areas were also observed in large vesicles or in the cell floor in as single layer beating areas. Picture 3 shows such beating areas. On the right side of picture there are two beating areas in EB medium, the black one is an aggregate and the clear one is single layer beating area. Beating area formed in a vesicle is on the left side of picture.



PICTURE 3. Beating areas from h1/12 p.28 and H7 p.60

5.1.2 Dissociated beating areas

The survival of dissociated cells was not thoroughly tested, but from visual observation it was clear that most of the dissociated cells did not survive the procedure. On the first day after dissociation there were a large number of dead cells floating in the medium. Right side of picture 4 shows some attached cells growing on the plate and multiple dead cells floating above them. The survived cells showed a good percentage of beating (results are only visual estimations) and the structure of cardiomyocytes was visualized from the single beating cells. On the left side of picture 4 is a good example of a single cardiomyocyte cell.



PICTURE 4. Dissociated cardiomyocytes from h1/12 p.30 cell line

5.2 Immunocytochemically stained cells

Each antibody yielded successful results by binding to their target area in each cell line. First pair of antibodies, Connexin 43 + α-Actinin showed cardiomyocytes stained red with Connexin and Ca2+-channels and Z-disc filaments stained green with α-Actinin, as seen in the figure 7 (pictures A2 and A2) which represents every antibody pairs. Pictures were not included of every possible cell line, passage and medium, because the beating cells were very similar in single cell state. In the second pair of antibodies, ventricular MHC stained the ventricular cardiomyocytes with green and Troponin T stained all cardiomyocytes with red (B1 and B2.) The last pair of antibodies visualized the amount of cardiomyocytes in the well compared to other cell types. Troponin T stained the cardiomyocytes red and Vimentin stained other cells green (C1 and C2.) The cell nuclei are stained blue in every picture.

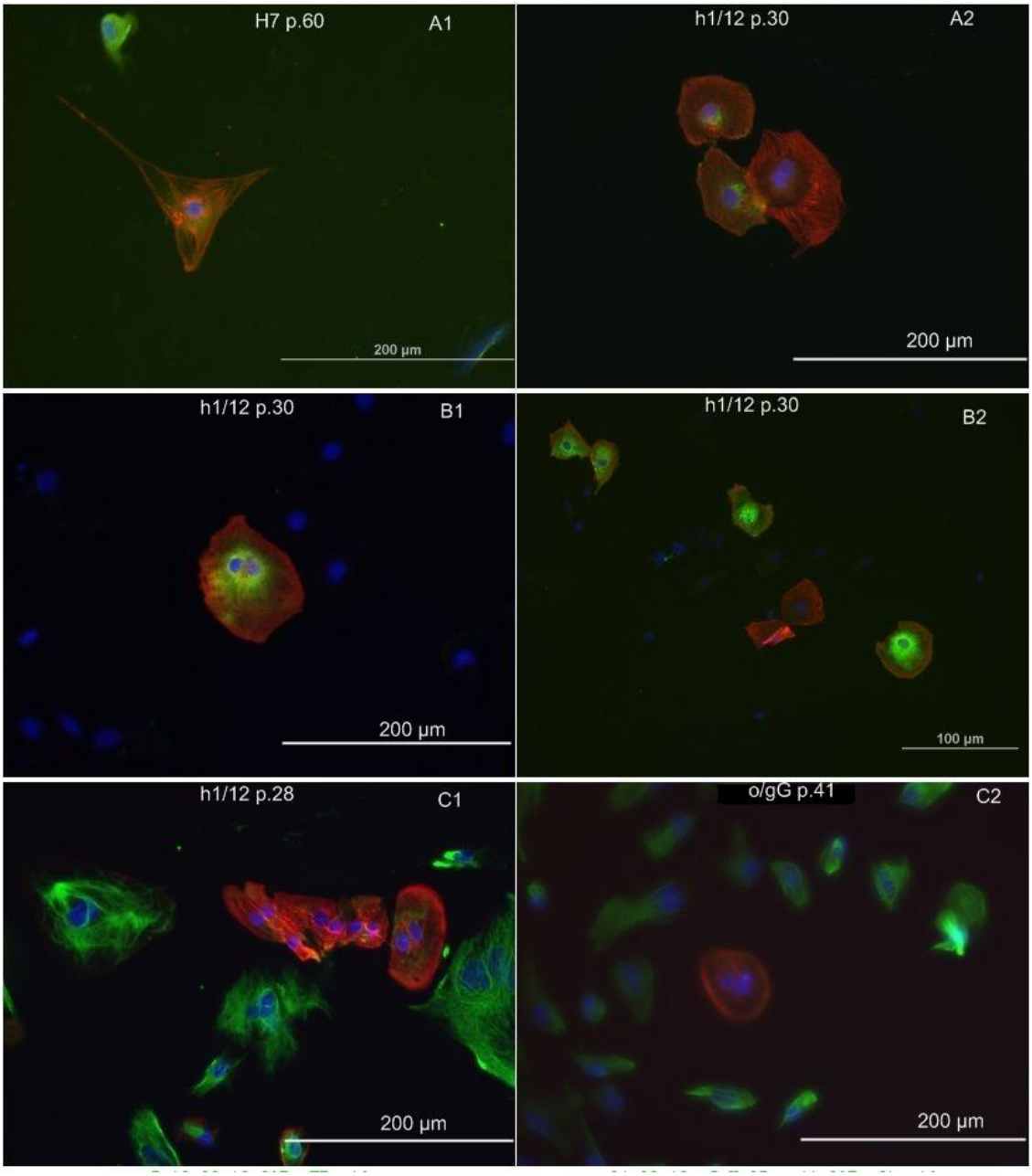


FIGURE 7. Immunocytochemical staining of cardiomyocytes

5.3 RNA extraction

RNA extraction was carried out successfully and almost every sample yielded satisfying amounts of RNA. The problems roused with day 0 samples and from differentiated cells grown in 0% KO-SR-medium. The RNA concentration in these samples was almost consistently two to ten times lower than in other samples. The purity of the RNA was

really good, the 260/280 ratio was near 2,10 in most samples, with the same exception of day 0- and 0%-samples. Tables 13 and 14 show the RNA concentrations and purities from each cell line, media and time points.

TABLE 13. RNA extraction results from first passages cell lines

RNA extraction	H7 p.57		h1/12 p.28		o/gG/25 p.41	
Sample	µg/µl	260/280	µg/µl	260/280	µg/µl	260/280
d0_1	84,96	1,72	39,6	1,88	31,85	2,1
d0_2	26,14	2,64	20,88	2,48	28,75	2,09
d13_1	114,52	2,14	290,24	2,14	97,73	2,2
d13_2	64,53	2,2	147,09	2,16	45,58	2,18
d22 0%_1	141,7	2,15	188,81	1,89	49,11	2,24
d22 0%_2	131,24	2,02	181,45	2,07	23,11	2,59
d22 10%_1	261,83	2,09	250,81	2,12	234,24	2,16
d22 10%_1	326,1	1,84	192,57	2,12	287,61	2,17
d22 EB_1	226,84	2	168,32	2,08	266,81	2,16
d22 EB_2	274,81	1,95	269,52	2	271,73	2,16
d22 Reges_1	220,12	2,16	109,85	2,1	153,89	2,16
d22 Reges_2	239,7	2,15	193,59	2,09	53,29	1,98
d30 0%_1	24,23	1,76	40,13	2,34	4,46	1,56
d30 0%_2	82,74	1,7	16,55	2,21	2,95	3,19
d30 10%_1	135,29	1,86	289,71	2,15	237,76	2,13
d30 10%_2	188,3	2,12	159,38	2,17	165,85	2,17
d30 EB_1	204,88	2,15	228,66	2,16	250,63	2,15
d30 EB_2	156,6	2,24	175,05	2,14	261,27	2,16
d30 Reges_1	162,28	2,11	219,1	2,17	93,92	2,2
d30 Reges_2	163,06	2,04	159,53	2,18	127,03	2,22

TABLE 14. RNA extraction results from second passage cell lines

RNA extraction	H7 p.60		h1/12 p.30	
Sample	µg/µl	260/280	µg/µl	260/280
d0_1	22,28	1,82	11,96	2,34
d0_2	15,75	2,16	-	-
d13_1	72,08	2,18	116,14	2,11
d13_2	75,56	2,18	56,89	2,28
d22 0%_1	40,05	2,23	12,46	1,95
d22 0%_2	25,42	2,51	21,37	2,09
d22 10%_1	59,01	2,17	126,66	2,17
d22 10%_1	59,17	2,14	265,3	2,16
d22 EB_1	192,31	2,13	74,39	2,16
d22 EB_2	282,09	2,15	101,92	2,15
d22 Reges_1	87,37	2,21	88,97	2,16
d22 Reges_2	121,56	2,17	60,96	2,2
d30 0%_1	2,25	1,52	11,79	1,98
d30 0%_2	1,89	0,96	2,35	2,82
d30 10%_1	161,5	2,13	123,48	2,15
d30 10%_2	129,87	2,15	222,64	2,15
d30 EB_1	193,59	2,13	13,33	2,2
d30 EB_2	77,82	2,14	238,55	2,15
d30 Reges_1	-	-	351,27	2,21
d30 Reges_2	-	-	49,03	2,23

5.4 Q-PCR results

Stem cells from tested cell lines and cardiomyocytes differentiated in four different media were tested for the gene expression of certain stem cell- and cardiac-specific markers using quantitative polymerase chain reaction. The method showed that the expression of stem cell markers Nanog and OCT3/4 (POU5F1) was very high in undifferentiated cell samples taken in day 0 and close to non-existent in differentiated cardiomyocytes. Figure 8 shows the expression of tested markers Nanog and POU5F1. The height of bars represents the relative gene expression of the samples in relation to each other. The bars are similar in day 0 because the medium is the same in every sample at that point; the new test medium was changed only on day 15. The black line at the top of the bars represents the error marginal, which comes from combining the triplicate samples.

The expression of cardiac markers showed more or less the desired gene expression. Each marker was expressed much stronger in cardiomyocytes from day 13-, 22- and 30-samples than in stem cells from day 0 samples. Each day 13 sample is the same, which is the reason for identical gene expression bars at day 13. The most distinguishable was the results from 0% KO-SR medium. The relative gene expression bar was much higher at day 30 samples than in any other of the three media. Figures 9 and 10 show how the gene expression increases even ten-fold in day 30 samples in 0%-medium, compared to day 22 samples. Similar expression at day 30 did not appear in any other tested media.

Out of the three tested cardiac markers, ISL1expression was the highest in day 13 samples and NKX2,5 and Troponin T very expressed more in day 22 and 30 samples. There were exceptions also, most likely caused by the differences in cell lines and the randomness of samples taken for the q-PCR.

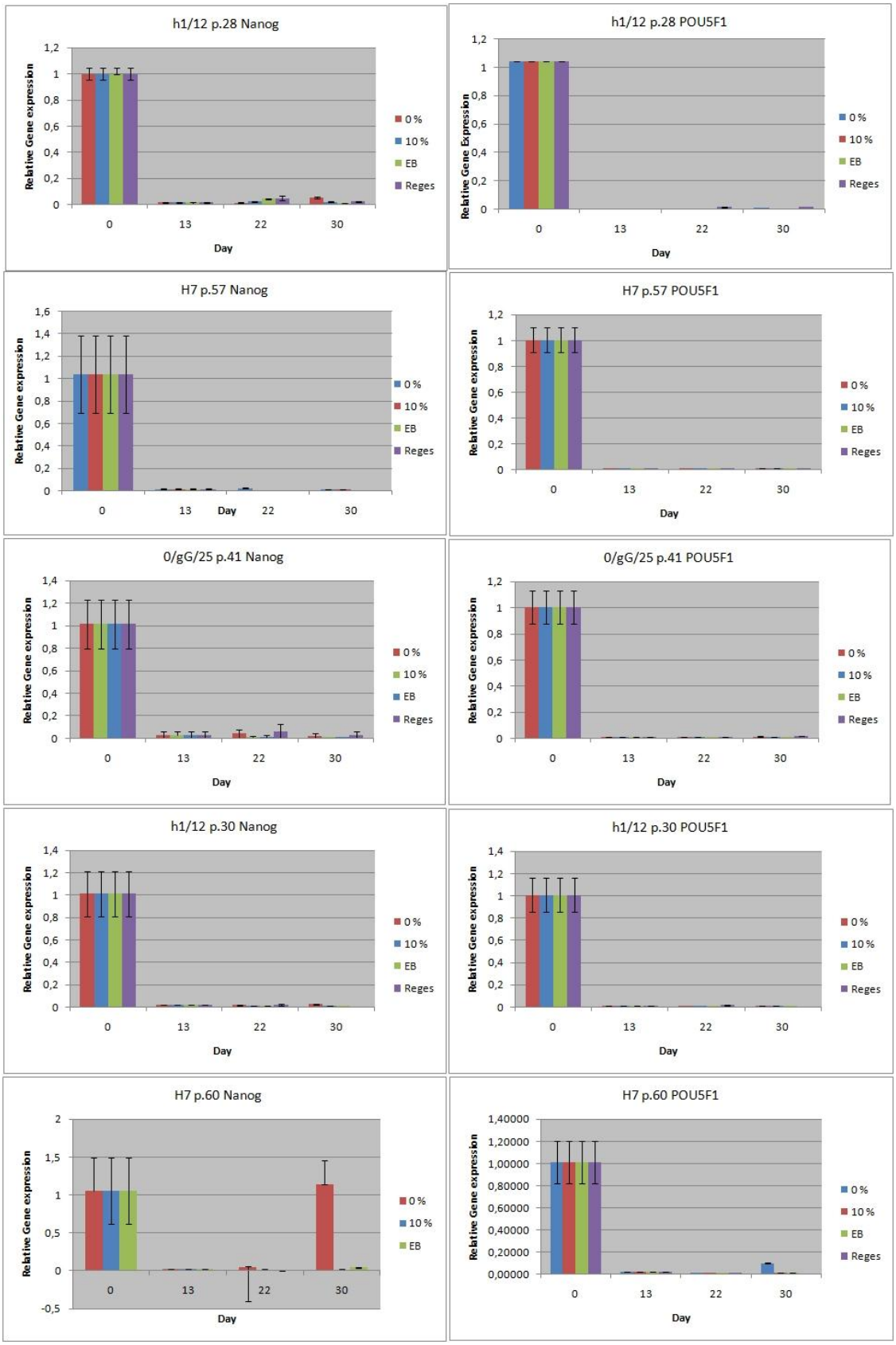


FIGURE 8. Gene expression of Nanog and POU5F1 (OCT3/4) markers

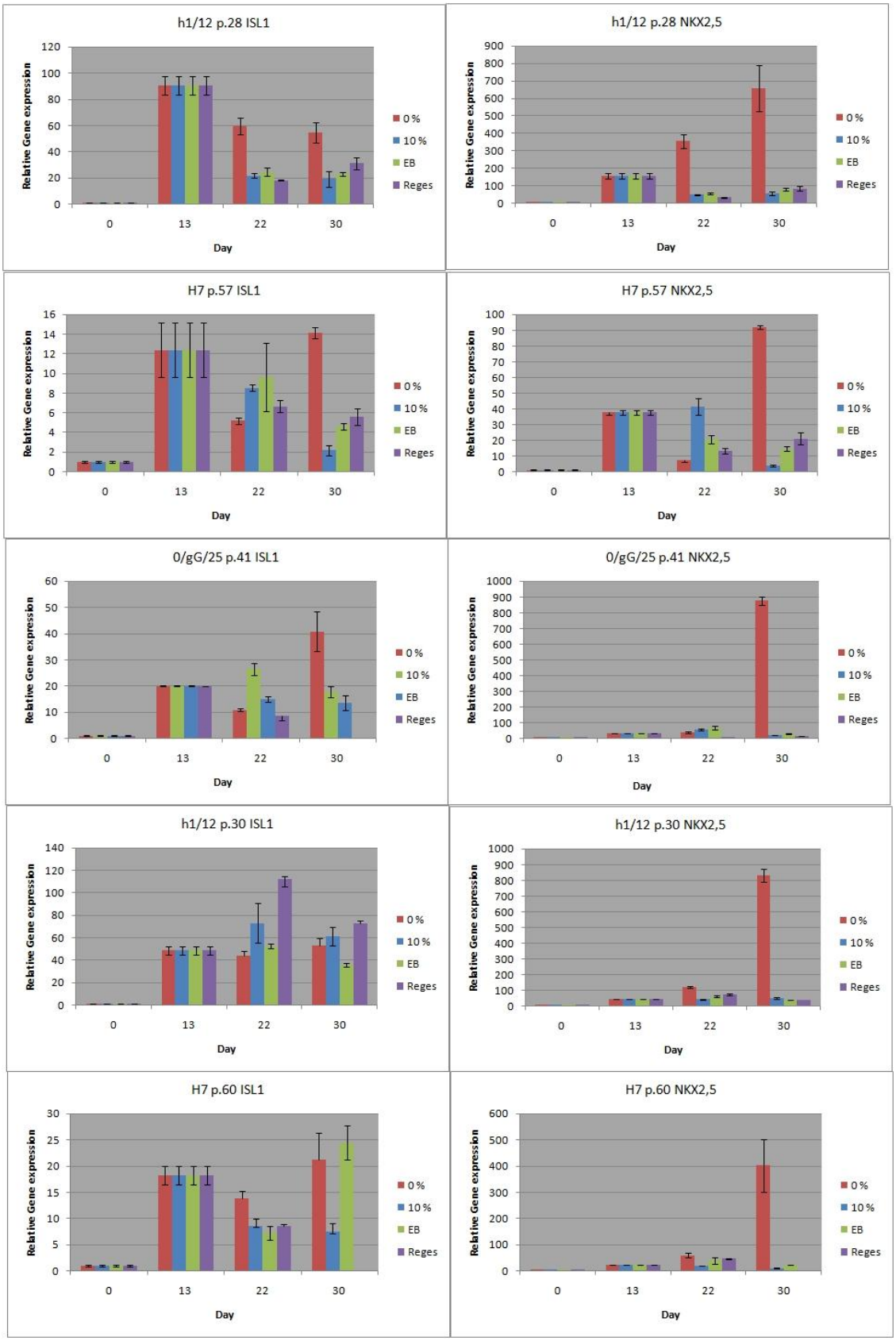


FIGURE 9. Gene expression of ISL1 and NKX2,5 markers

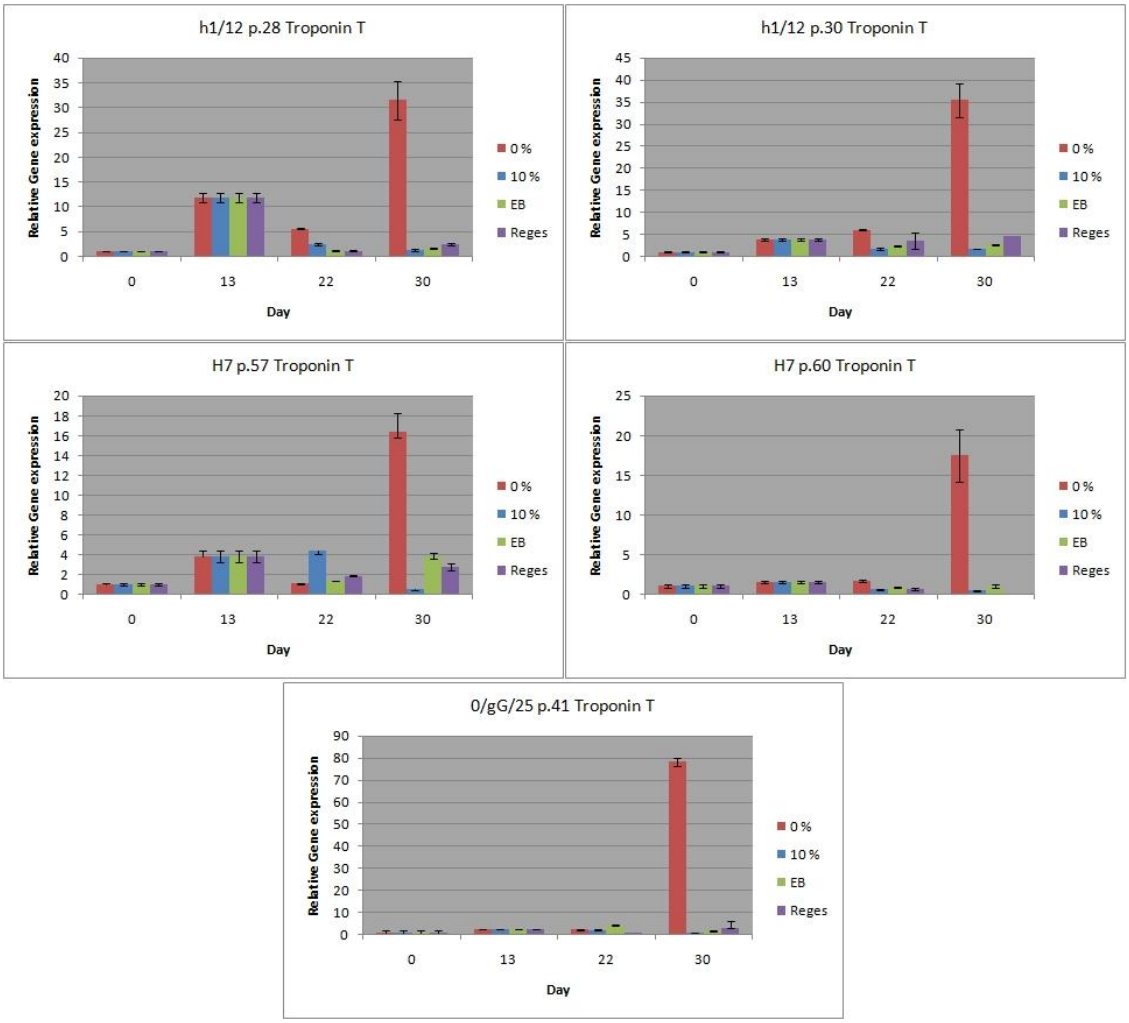


FIGURE 10. Gene expression of Troponin T-marker

In the q-PCR results, there does not appear to be any clear difference between the cell lines, whether they were iPS cell lines or not. ISL1 markers shows best this seemingly random gene expression of different samples. The same randomness occurs between different passages of the same cell line. For example in h1/12 p.28 the expression of ISL1 is low on day 22 and 30 Reges samples, but much higher in p.30 samples. Only medium that shows constantly high gene expression is 0% KO-SR, in day 30 samples.

6 DISCUSSION

In the study a successful differentiation in form of beating areas was acquired with each medium, and only a small difference in the differentiation percentage between test media was observed. The best medium in this sense was 0%-KO-SR hes medium. The results were interesting, because this medium yielded fine beating areas but almost all other cell mass detached from the growing plate. This could help the dissociation of beating areas considerably in the future, but it could also affect the maturation of cardiomyocytes negatively. This study does not show any signs of such effects though. A very useful point for future research is that 0%-KO-SR-medium is xeno- and animal free and could thus be used in clinical applications.

Media that used serum extracted from animals (EB) and media with serum replacement (10% KO-SR hes) worked almost as well as 0%-medium and should not be neglected in the research, at least yet. Still, it was good to get similar results as Passier & Co (2005) in their article about cardiomyocyte differentiation in serum-free culture. The most worrisome was the unstable beating of cells in Reges medium. This kind of beating is not desirable for cardiomyocytes, when considering future clinical applications. Because the medium reagents are known, and it mostly resembles knockout serum replacement formulation, it is fairly easy to guess what reagent caused the instability. FGF basic and Activin-A are reagents that have been used for a long time in cardiac differentiation, so the possible cause is retinol. It would be interesting to see whether Reges would yield similar or completely different results without retinol or modified some other way.

It was expected that the H7 control cell line would yield best differentiation results, but between iPS cell lines, the one derived from a new-born fibroblasts (h1/12) seemed to fare better than patient iPS line (0/gG/25). The reasons for this are numerous and mostly unknown and require more research. The obvious decline in the amount of beating areas between first and second passages of all cell lines is probably caused by the problems faced at Regea with the cell growing plates. The batch of plates used during second passages was apparently flawed somehow and could have affected the growth of cells negatively. Small calculation errors may have also been made during the microscoping

of beating areas, but every plate was counted with the same systematical method allowing no bias towards any medium or cell line. The q-PCR results in part also confirm the differences between the cell lines, showing slightly different gene expression between cells lines with the same medium.

The immunocytochemical staining showed the successful differentiation from each cell line and medium and also showed that stem cells differentiated into ventricular and atrial cardiomyocytes, as shown in Humpath (2010). The high amount of non-cardiomyocytes in the beating areas was also visualized with Vimentin-antibody.

RNA yield from commercial RNA extraction kit protocol was good enough for most samples. The goal was to acquire 1mg of RNA from the duplicate samples to be used in the cDNA reverse transcription. The low amount of RNA in day 0 samples is hard to explain, but the loss of most cells in 0%-medium wells was the cause for low RNA yield. All the samples were taken randomly, so some samples may have had much less cell growing in the wells. The purity of the samples was good, excluding the previous day 0- and 0%-samples. Despite low RNA concentrations, each sample produced enough cDNA for q-PCR analysis.

Quantitative PCR tested the samples for their expression of certain genes typical for either stem cells or cardiomyocytes. Results were very successful for both markers. As described by Sperger & Co (2003), the stem cell markers Nanog and OCT3/4 were clearly expressed only in day 0 samples, except for the H7 p.60 day 30 sample in 0%-medium, where Nanog is expressed higher than in day 0 samples. The same sample expresses cardiac markers also very highly, so it may be that the sample for Nanog analysis was contaminated.

The expression of cardiac markers ISL1, NKX2,5 and Troponin T in differentiated cardiomyocytes was higher than in stem cell samples, as expected. Cai & Co (2003) described ISL1 as a cardiac mesoderm marker, so the gene expression was expected to be highest in the early stage of differentiation, in this case on day 13. The results confirmed this with ISL1 expression being highest in day 13 in most cell lines. The other markers, NKX2,5 and Troponin T provided higher gene expression in older samples as suggested by Megy & Co (2002) and Schwartz & Co (1999).

Particularly interesting were the results from 0% KO-SR medium. By day 30, the cardiomyocytes seemed to be the most matured in this medium, when comparing the gene expressions of all three tested cardiac markers to other test media. This suggests that 0% KO-SR is the best suited for long term differentiation protocol. The unusually high gene expression may be explained with the low yield of RNA from 30 day samples in 0% KO-SR-medium. The starting material was small, but contained more cardiomyocytes than any other media-sample, because other than beating cells had detached from the wells. The RNA material was amplified as cDNA before q-PCR and because other samples had more RNA from non-cardiomyocytes, the relative amount of cardiomyocyte cDNA was higher in day 30 0% KO-SR samples.

The results from q-PCR are indicative about the cardiac differentiation, but the technique is not absolutely accurate. It would have been interesting to verify the q-PCR results with protein analysis, using western blot technique to accurately identify target proteins from sample cardiomyocytes. Western blot as an additive analysis is recommended in the future research.

This study was done to test the cardiac differentiation capabilities of four media: 0% KO-SR hes, 10% KO-SR hes, EB-medium and Reges-medium. Out of these four 10% and EB medium worked fine in differentiation, producing nicely beating cardiomyocytes with the desired gene expressions. Reges-medium worked also in differentiation, but the instability in the beating of cells makes it undesirable for research at least until the cause is better know. Based on the results of this study the medium best suited for cardiomyocyte differentiation seems to be 0% KO-SR hes-medium. Not only did it produce the most beating areas with good morphology and beating, but the maturation of cardiomyocytes in the late stage of differentiation was much better than with other media.

One aim of the study was also to find a cheaper medium for cardiac differentiation in Regea. Since 0% KO-SR hes is the most plain of the media used in the study, it’s also the cheapest. And as the results suggest this medium as the most suitable for cardiomyocyte differentiation, the other aim was also fulfilled. Based on these results this medium definitely deserves additional research, perhaps on even older than 30-days-old cardiomyocytes.

The understanding and optimization of the mechanism of cardiomyocyte differentiation from pluripotent stem cells is one step towards the solution of treating cardiac diseases. The introduction of induced pluripotent stem cells helped study cardiac diseases but the whole differentiation mechanism is still not clear enough to study clinical applications. With the knowledge of this study, the understanding of certain effects of culture media and reagents in the differentiation characteristics of cardiomyocytes can be used in additional studies to improve cardiomyocyte differentiation.

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LIST OF REAGENTS USED IN CARDIOMYOCYTE DIFFERENTIATION MEDIA

APPENDIX 1: 1 (2)

0% KO-SR hes

Reagent	Amount in 50ml
KO-DMEM	48,75ml
NEAA	0,5ml
Glutamax	0,5ml
Pen/Strep	0,25ml
b-mercaptoethanol	97,5µl

10% KO-SR hes

KO-SR	5ml
KO-DMEM	43,75ml
NEAA	0,5ml
Glutamax	0,5ml
Pen/Strep	0,25ml
b-mercaptoethanol	97,5µl

EB-medium

KO-DMEM	39,75ml
FBS	10ml
NEAA	0,5ml
Glutamax	0,25ml
Pen/Strep	0,25ml

Abbreviations:

NEAA: non-essential amino acids

FBS: Fetal bovine serum

Pen/Strep: Penicillin and Streptomycin

APPENDIX 1: 2 (2)

Reges-medium Component	Concentration mg/l
Amino acids	
Glycine	53
L-histidine	183
L-isoleusine	615
L-methionine	44
L-phenylalanine	336
L-proline	600
L-hydroxyproline	15
L-serine	162
L-threonine	425
L-tryptophan	82
L-tyrosine	84
L-valine	454
Vitamins	
Thiamine	9
Retinol	0,5
Antioxidants	
Glutathione	1,5
Ascorbic acid	50
Proteins	
Human serum albumin	10000
Transferrin	8
FGF basic	0,008
Activin A	0,005
Trace elements	
Trace element B	1 / 1000
Trace element C	1 / 1000
Selenium	0,00001
Other components	
Non-essential amino acids	1 %
L-glutamine	2mM
b-mercaptoethanol	0,14mM

LIST OF DISSOCIATION BUFFER REAGENTS APPENDIX 2

Dissociation buffers Reagent	**Buffer 1 (100ml)**	**Buffer 2 (100ml)**	**Buffer 3 (100ml)**
NaCl	12ml (1M)	12ml (1M)	-
CaCl2			-
K2HPO4	-	3µ (1M)	3ml (1M)
KCl	0,54,l (1M)	0,54ml (1M)	8,5ml (1M)
Na2ATP	-	-	2mmol/L
MgSO4	0,50ml (1M)	0,50ml (1M)	0,50ml (1M)
EGTA	-	-	0,1ml (1M)
Na Pyruvaat	0,50ml (1M)	0,50ml (1M)	0,50ml (1M)
Glucose	2ml (1M)	2ml (1M)	2ml (1M)
Creatine	-	-	5ml (1M)
Taurine	20 ml (1M)	20 ml (1M)	20 ml (1M)
Collagenase A	-	1mg/ml	-
HEPES	1ml (1M)	1ml (1M)	-
pH corr.	NaOH	NaOh	-
pH	6,9	6,9	7,2

LIST OF ANTIBODIES AND THEIR DILUTIONS APPENDIX 3

Primary Antibodies + wavelength (nm)	Origin	Dilution
Connexin 43	Rabbit	1 / 1000
α-actinin	Mouse	1 / 500
MHC	Mouse	1 / 100
Troponin T	Goat	1 / 500
Vimentin	Rabbit	1/1
Troponin T	Mouse	1 / 500
Secondary antibodies	Origin	Dilution
anti-mouse Alexa 568	Donkey	1 / 300
anti-rabbit Alexa 488	Donkey	1 / 300
anti-mouse Alexa 488	Donkey	1 / 300
anti-goat Alexa 568	Donkey	1 / 300
anti-rabbit Alexa 488	Donkey	1 / 300
anti-mouse Alexa 568	Donkey	1 / 300

RNA CONCENTRATIONS APPENDIX 4: 1 (2)

The RNA concentrations and amounts of samples pipetted for cDNA reverse transcription reaction. The total volume of 10µl was filled with RNase-free water.

Sample	**H7 p.57**		**H7 p.60**		**h1/12 p.28**		**h1/12 p.30**	
	µg/µl	sample µl	µg/µl	sample µl	µg/µl	sample µl	µg/µl	sample µl
d0_1	84,96	**5**	22,28	**5**	39,6	**10**	11,96	**10**
d0_2	26,14	**5**	15,75	**5**	20,88	**0**		
d13_1	114,52	**7**	72,08	**5**	290,24	**2**	116,14	**7**
d13_2	64,53	**3**	75,56	**5**	147,09	**3**	56,89	**3**
d22 0%_1	141,7	**4**	40,05	**10**	188,81	**2,4**	12,46	
d22 0%_2	131,24	**4**	25,42		181,45	**3**	21,37	**10**
d22 10%_1	261,83	**2**	59,01	**5**	250,81	**2**	126,66	**4**
d22 10%_1	326,1	**1,5**	59,17	**5**	192,57	**2,6**	265,3	**2**
d22 EB_1	226,84	**2**	192,31	**3**	168,32	**2,7**	74,39	**1**
d22 EB_2	274,81	**2**	282,09	**1,5**	269,52	**2**	101,92	**9**
d22 Reges_1	220,12	**2,3**	87,37	**3**	109,85	**3**	88,97	**10**
d22 Reges_2	239,7	**2**	121,56	**6**	193,59	**4**	60,96	
d30 0%_1	24,23	**5**	2,25	**5**	40,13	**10**	11,79	**10**
d30 0%_2	82,74	**5**	1,89	**5**	16,55	**0**	2,35	
d30 10%_1	135,29	**3,2**	161,5	**3**	289,71	**2**	123,48	**4,5**
d30 10%_2	188,3	**3**	129,87	**4**	159,38	**2,7**	222,64	**2**
d30 EB_1	204,88	**2,5**	193,59	**3**	228,66	**2**	13,33	
d30 EB_2	156,6	**3**	77,82	**5,5**	175,05	**3**	238,55	**4,2**
d30 Reges_1	162,28	**3,2**			219,1	**2**	351,27	**3**
d30 Reges_2	163,06	**3**			159,53	**3,5**	49,03	**0**

APPENDIX 4: 2 (2)

Sample	**0/gG/25 p.41**	
	µg/µl	sample µl
d0_1	31,85	**5**
d0_2	28,75	**5**
d13_1	97,73	**5**
d13_2	45,58	**5**
d22 0%_1	49,11	**5**
d22 0%_2	23,11	**5**
d22 10%_1	234,24	**2**
d22 10%_1	287,61	**2**
d22 EB_1	266,81	**2**
d22 EB_2	271,73	**1,7**
d22 Reges_1	153,89	**5**
d22 Reges_2	53,29	**4**
d30 0%_1	4,46	**5**
d30 0%_2	2,95	**5**
d30 10%_1	237,76	**2**
d30 10%_2	165,85	**3,2**
d30 EB_1	250,63	**2**
d30 EB_2	261,27	**2**
d30 Reges_1	93,92	**5,5**
d30 Reges_2	127,03	**4**